Artemia with Synbiotics Enrichment Improves Resistance Against *Vibrio parahaemolyticus* AHPND of *Litopenaeus vannamei* Larvae

Ervia Yudiati[1]*, Zaenal Arifin[2], Adi Santoso[1], Jelita Rahma Hidayati[3], Rabia Alghazeer[4], Nuril Azhar[1]

[1]Departement of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro
Jl. Prof. Jacub Rais, Tembalang, Semarang, Jawa Tengah 52075 Indonesia
[2]Fisheries Research Centre, Earth and Marimite Organization Research, National Research and Innovation Agency
CSC-BG LIPI Jl. Raya Bogor Km 46, Cibinong, Bogor, Jawa Barat, 16915 Indonesia
[3]Department of Marine Science, Faculty of Marine and Fisheries Sciences, Raja Ali Haji Maritime University
Jl. Politeknik Senggarang Tanjung Pinang, Riau Islands, Indonesia
[4]Department of Chemistry, Faculty of Sciences, University of Tripoli
Tariq Sayyidi al Misri Road, Tripoli, Libya
Email: eyudiati@gmail.com

Abstract

Shrimp, a high-protein food commodity, is one of the world's fastest-growing food-producing sectors. The present research aimed to find out the survival and growth of *Litopenaues vannamei* Post Larva (PL) and the resistance against VpAHND and stress salinity. 1500 PL were reared to two sets of experiments at the density of 50 PL. L^{-1}. The first set is purposed to determine the PL growth, resistance to *Vibrio paramaemolyticus* AHPND challenge and stress salinity. The second set is purposed to determine the survival rate. A Completely randomized design (CRD) with five treatments and three replications was conducted. The treatments are Artemia enrichment with different Alginate doses and probiotics (400, 600, 800 ppm Alg+pro), probiotics (Pro), and control without any synbiotics addition. PL was reared in 14 days. The survival rate, and weight gain were calculated. At the end of the experiment, 10 PL was challenged against VpAHPND at 1 x 10^7 CFU mL^{-1} by immersion methods. Twenty PL was exposed to stress salinity and shocked from 25 ppt to 0 ppt. The best survival rate ((78±2%), and tolerance to osmotic stress was reached at PL fed on a combination of alginate and FNCC-002 *Lactobacillus bulgaricus* probiotics ($p<0.05$). PL fed on Artemia enriched probiotics reached the highest resistance to severe VpAHNPD. The weight gain among treatments is similar. It can be concluded that synbiotics of alginate as prebiotics and FNCC-004 probiotics work synergically and this might be interrelated with immune response.

Keywords : Artemia, Alginate, Shrimp, Vibrio spp.

INTRODUCTION

Litopenaeus vannamei is one of the main part aquatic products among fishery trading commodities globally, and the most important species of shrimp culture in Indonesia. The fact of reducing wild-capture harvest i.e. 3.1 million tonnes of *L. vannamei* farming reported nearly 6.5 million tonnes (56%) from the global shrimp production in 2019 (FAO, 2021).

In practice, one of the most important biosecurity aspects for enhancing early culture stages including post larva and providing an effective shrimp farming grow-out phase is to find out high-quality rearing of post larva (PL) shrimp throughout the nursery phase (Rodríguez-Olague *et al*., 2021). The success of nursery phase depends on rigorous organize to adequate feeding, water quality, and other specialized management (García-Guerrero *et al*., 2015). Particularly, the shrimp PL stage needs a highly nutritious diet throughout nursery rearing that occupies high quality marine ingredients (Ayisi *et al*., 2017). Artemia nauplii is a vital crustacean larval live food that can be cultured in huge numbers at large densities (Sorgeloos, 1980). However, Artemia nauplii cannot meet up the nutritional supplies for larval development when used as the singular food. Several researchers has been reported Artemia enrichment by carotenoid-rich microalgae (Gui et el., 2022), *Dunaliella salina* (Bhuvaneshwari *et al*.,

*Corresponding author
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2018) and *Isochrysis galbana* (Martelli *et al*., 2020) which delivered the basic nutrients for the growth and development of nauplii.

Shrimp production in Indonesia was 786,654 tonnes (2015), and declined to 265,000 tonnes (2016) (Febriani *et al*., 2018), mainly due to disease problems (Trang *et al*., 2019), especially Vibriosis caused by *Vibrio parahaemolyticus*, AHPND strain (Kumar *et al*., 2020), even at PL stage (Yu *et al*.,_2022). Recent research discovered that *V. parahaemolyticus* AHPND strain, carries the specific the pirA-, pirB- genes that contain an extracellular toxin. This highest lethal strain is the early *L. vannamei* juvenile mortality syndrome which caused 100% mortality within 24 hours of infection (Choi *et al*. 2017).

The health status of shrimp and aquatic animals is closely interrelated to gut microbiota and the environment (Lima *et al*., 2021). Valuing the effects of feeding trials on the performance of PL shrimp not only assesses their growth and survival but also their quality through *V. parahaemolyticus* AHPND challenge and a stress test (Yudiati *et al*., 2020).

Alginate, the water extracted from *Sargassum* sp cell wall is well known as immunostimulant agent (Isnansetyo *et al*., 2015; Yudiati *et al*., 2016; Yudiati *et al*., 2019) and worked synergically as synbiotics with *Lactobacillus bulgaricus* probiotics (Yudiati *et al*., 2021). Furthermore, Du *et al*. (2019) denoted that *Lactobacillus pentosus* is able to increase disease resistance, gut bacterial diversity, and growth performance. We have evaluated that sodium alginate has a good potency as synbiotics and had a synergetic mechanism with *L. bugaricus* probiotics to counteract the combat of three single and combined *Vibrio* spp. in Artemia as biomodel (Yudiati *et al*., 2021). The key to this mechanism is the enhance of the immune system and the balance of microbiota. Gut microbiota could provide a blockade against pathogen invasion, start promoting host nutrient absorption through extracellular product secretion, and accelerate immune response (Libertucci & Young, 2019).

Salinity variation and fluctuation, especially in tropical areas is risky, due to rain and precipitation. Among the stress test methods, the assessment of resistance to salinity shock is suitable and effective tool for evaluating the quality of penaeid PL, particularly in tropical area such as Indonesia.

There is still lack of current information concerning the performance *L. vannamei* PL fed on Artemia enriched with combination of sodium alginate and probiotics which then followed by challenged against high virulence *V. parahaemolyticus* AHPND as well as stress salinity. This study is aimed to find out the survival and growth of *L vannamei* PL and the resistence against VpAHND and stress salinity.

MATERIAL AND METHODS

Production of Sodium Alginate (Alg) from *Sargassum sp*

This research was done from July to September 2022. The extraction and production of sodium alginate were basically based on Yudiati et al., (2016). Four grams of dried *Sargassum* sp. was added to 100 mL aquadest, 5 g Na_2CO_3, dan 1.86 g EDTA and mixed. HCl was added to manage the desirable pH, stirred and filtered. The KCl was then put into the filtrate and followed by absolute ethanol for precipitation. Centrifugation was then administered for pellet production and dried up. Our sodium alginate was fit to the standard alginate (Sigma®, USA) (Yudiati *et al*., 2016), with 217.5 KDa molecular weight at 89,95% acetylation degree (Yudiati *et al*., 2018).

Preparation of *L. bulgaricus* FNCC–004 Probiotics and sodium alginate

FNCC–004 was provided by Tropical Marine Biotechnology Lab., Dept. of Marine Science, Diponegoro University. Prior to these, all glassware, media and materials were sterilized using an autoclave and spraying with 70% alcohol inside Laminar Air Flow under UV exposure (Guridi *et al*., 2019).

One ose of FNCC–004 cultured in de Man, Rogosa dan Shape Agar/MRS Agar media was suspended to 100 mL Nutrient Broth/NB (Merck, USA) and 5% of MRS broth media dan incubated for 24 hrs at 37ºC (Yudiati *et al*., 2020). The synbiotics were prepared by mixing the FNCC–004 and sodium alginate. A serial concentration of alginate 0, 400, 600, dan 800 ppm was adjusted by adding 0.006, 0.012, and 0.018 g Alginate and diluted into 20 mL liquid culture of FNCC–004 dan 180 mL sterile sea water. The FNCC–004 and alginate fermentation was done by stirring at 150 rpm at room temperature (Yudiati *et al*., 2021).

Nauplii Artemia Enrichment

One gram of Artemia cyst (Supreme Plus®, Golden West Artemia) was weighted and hatched

in strong aerated 1,000 mL sterile seawater. Along 16-18 hrs nauplii was hatched, collected, and ready to use (Rudtanatip *et al*., 2019). The enrichment of Artemia nauplli was done by immersion. 250-500 nauplii Artemia was immersed in 50 mL fermented probiotics and alginate for one hour (Yudiati *et al*., 2021). The enriched Artemia is ready to use as natural feed for *L. vannamei* PL.

Experimental Design and *L. vannamei* Post Larva Feeding Trial

This is an experimental laboratory research. A Completely randomized design (CRD) with five treatments and three replications was conducted. The treatments are Artemia enrichment with different Alginate dose and probiotics (400, 600, 800 ppm Alg+pro), probiotics (Pro), and control without any synbiotics addition

We have done the experiment in two sets. First set to determine growth performance, bacterial challenged and stress salinity, and another set to determine survival rate. 1,500 *L. vannamei* PL (0.01 mg) were acclimated in 45 L container with 30 L in volume and was adapted with 25 ppt seawater for 24 hrs. Firstly, one set trial of growth performance, bacterial challenged and stress salinity was prepared. In next day, 750 PL were selected and stocked to round bottom flask (1,000 mL in volume) with 25 ppt seawater media and started feeding on the enrichment Artemia according to the treatments. The density of each flask was 50 PL. The rearing period is 14 days and the feeding frequency was five times/day (07.00, 10.00, 13.00, 16.00, dan 19.00). To maintain the water quality, the shrimp faecal was siphoned every two days. The water exchanged was 30% from the total volume. At 14 days of rearing, 5 PL were picked up randomly and observed under microscope to assess the PL weight. Other 10 PL was challenged against *Vibrio parahaemolyticus* AHPND and 20 PL for salinity stress with 0 ppt salinity exposure. Another similar set of experiment was applied to determine the PL survival rate. The survival rate was counted at the end of experiment (14 days).

Preparation of *Vibrio parahaemolyticus* AHPND and Challenge Test

V. parahaemolyticus (VpAHPND) strain was purchased from Main Center of Brackishwater Aquaculture, Jepara. *VpAHPND* was recultured in laboratory and stocked Alkaline Peptone Water (APW, Merck), and followed by culturing in liquid APW and left for 24 hrs to grow, then followed by centrifugation at 4,200 rpm, for 15 minutes. Sterile seawater was used for pelleted bacterial cell dilution. In terms of bacterial challenged to PL, the density of *VpAHPND* was evaluated and determined in spectrophotometer at 600 nm. This 2.0 optical density is equal to 1 x 10^9 CFU mL^{-1} (Kongchum *et al*., 2022).

The challenge methods was modified from Balcázar *et al*. (2007). Ten PL was separated to the new rounded bottom flask and set up according to the treatments. Flasks were previously filled with 99 mL sterile seawater. One mL *VpAHPND* (1 x 10^7 CFU mL^{-1}) were introduced to the flasks. The survival rate was monitored every day until 7 days.

Stress Salinity Exposure Test

The stress salinity was modified from Richardson *et al*. (2021). At the end of experiment, 20 survived PL from 25 ppt seawater media was taken and moved to petridish (50 mL freshwater, 0 ppt) according to the treatments. The mortality of PL was monitored and counted every 10 minutes for 60 minutes exposure.

Data Analysis

The data was statistically analyzed using R-Studio to determine the differences between treatments. The data was confirmed by one-way analysis of variance (ANOVA) to determine whether the treatment had a significant effect ($p<0.05$). To determine the differences, LSD was applied for the survival rate, weight gain, *VpAHPND* challenge and stress salinity exposure test data.

RESULTS AND DISCUSSION

Results shows that *L. vannamei* PL fed on Artemia enriched with alginate and probiotics reach the best survival rate at (78±2%), follow with other treatments and control reach the lowest PL survival rate at (43±14.75%). (Figure 1).

Sodium alginate which extracted from *Sargassum* sp. cell wall is a polysaccharide and constructed with mannuronic and guluronic monosaccharides (Dragdet & Taylor., 2011). The chemical structure of polysaccharide from alginate is similar to the bacterial cell wall, so, therefore, as this structure is recognized by host receptors, the immune response is then started by haemocyte proliferation. Amparyup *et al*. (2013) noted that Phenol Oxidase is the key role of innate immune in shrimp. PO contributes in melanogenesis by

transforming phenols to quinones, and polymerizing to form melanin. Melanin assists in preventing pathogens from growing and reproducing. Other parameters on the rise of immune response were indicated by the increasing activity of two enzymes involved in Reactive Oxygen Species (ROS) scavenging namely Super Oxide Dismutase and Catalase to counter the production of radical species production caused by bacterial infection (Yudiati *et al*., 2019, Messina *et al*., 2014). ROS is beneficial to the immune system, but excessive amounts can be harmful (Cheng at al., 2004). As the immune is getting high, the gene-related immune expression (Pro PhenolOxydase, β, 1-3 Glucan Binding Protein and Lectin) was also upregulated (Yudiati *et al*., 2019; Xing *et al*., 2020). As a result, the PL survival fed on Artemia enriched with 400 ppm alginate and *L. bulgaricus* probiotics reached the best one, in contrary compared to control. The synbiotics is in dose dependant manner.

Lactic acid bacteria (*L. bulgaricus*) as probiotics secreted exopolysaccharides (Daba *et al*., 2021). In their works Du *et al*. (2019) reported that *Lactobacillus pentosus* is able to increase disease resistance, gut bacterial diversity, and growth performance. Similar to this research, Roomiani *et al*. (2019) reported that application of *L. bulgaricus* in the diet of *L. vannamei* significantly boosted the immune parameters namely THC, PO, and respiratory burst activity (RBA). Healthy and balance gut microbiota could provide a blockade against pathogen invasion, start promoting host nutrient absorption through extracellular product secretion, and accelerate immune response (Libertucci & Young., 2019). Garces *et al*., (2015) noted that *L. pentosus* H 15 outcompeted the pathogenic *V. alginolyticus.* The lactic acid bacteria is selectively stick on to mucosal surfaces, create cell-bound biosurfactants, and dislocating pathogenic strains.

Figure 2 shows that *L. vannamei* PL fed on Artemia enriched with probiotics, alginate and/or FNCC-004 probiotics are similar in terms of weight gain.

This data suggested that the innate immune which boost by alginate is characterised by fast response immune and reach the peak at 14 days (Yudiati *et al*., 2019; Cheng *et al*., 2005). Since this works was conducted in 14 days, the weight gain was not affected specifically. Though, all PL fed on enriched Artemia with alginate and probiotic treatments tend to be higher than control. Our study revealed that *L. vannamei* grow out shrimps fed on alginate supplementation resulted better growth than control (Yudiati *et al*., 2016). Unfortunately, so far, there is no specific information regarding to the growth of shrimp fed on synbiotics alginate and lactic acid bacteria.

Figure 3 shows *L. vannamei* PL survival rate fed on Artemia enriched with probiotics, alginate and/or FNCC-004 probiotics at 7 days observation after *VpAHPND* challenge. In general, all treatments are similar at early days after challenge and late days after challenge. It is interesting to highlight, at 2,3, and 4 days after immersion

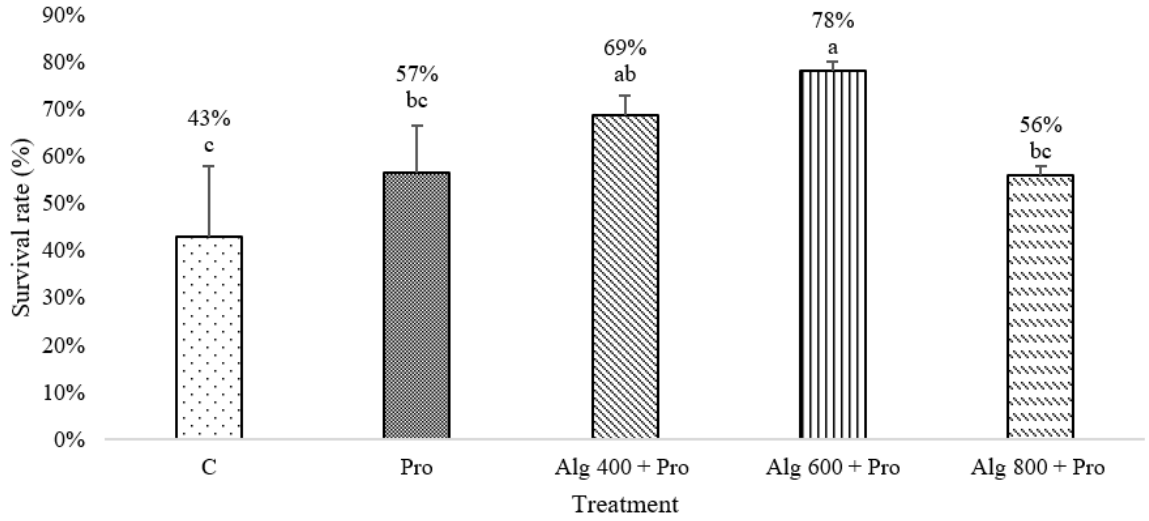


Figure 1. Survival rate of post larva *L. vannamei* at 14 days of rearing fed on different Artemia enrichment. Different letter denotes significantly different at $p<0.05$.

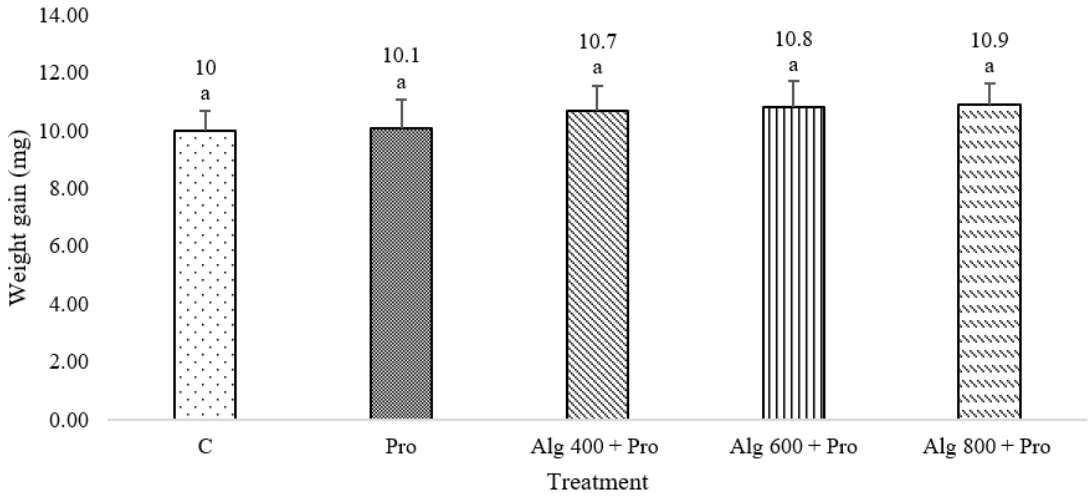
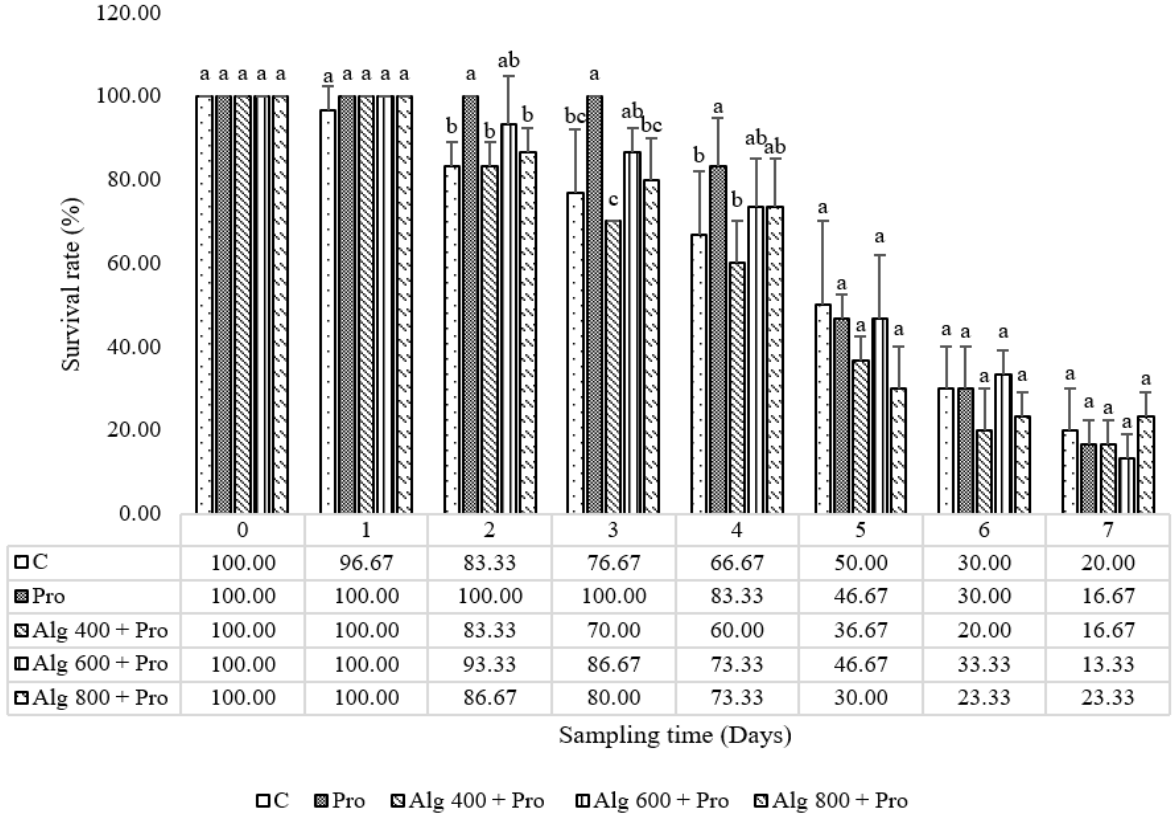


Figure 2. Weight gain of post larva *L. vannamei* at 14 days of rearing fed on different Artemia enrichment. Different letter denotes significantly different at p<0.05



	0	1	2	3	4	5	6	7
C	100.00	96.67	83.33	76.67	66.67	50.00	30.00	20.00
Pro	100.00	100.00	100.00	100.00	83.33	46.67	30.00	16.67
Alg 400 + Pro	100.00	100.00	83.33	70.00	60.00	36.67	20.00	16.67
Alg 600 + Pro	100.00	100.00	93.33	86.67	73.33	46.67	33.33	13.33
Alg 800 + Pro	100.00	100.00	86.67	80.00	73.33	30.00	23.33	23.33

Figure 3. Survival rate of *L. vannamei* PL fed on different Artemia enrichment after 7 days of VpAHPND challenged. Different letter denotes significantly different at p<0.05

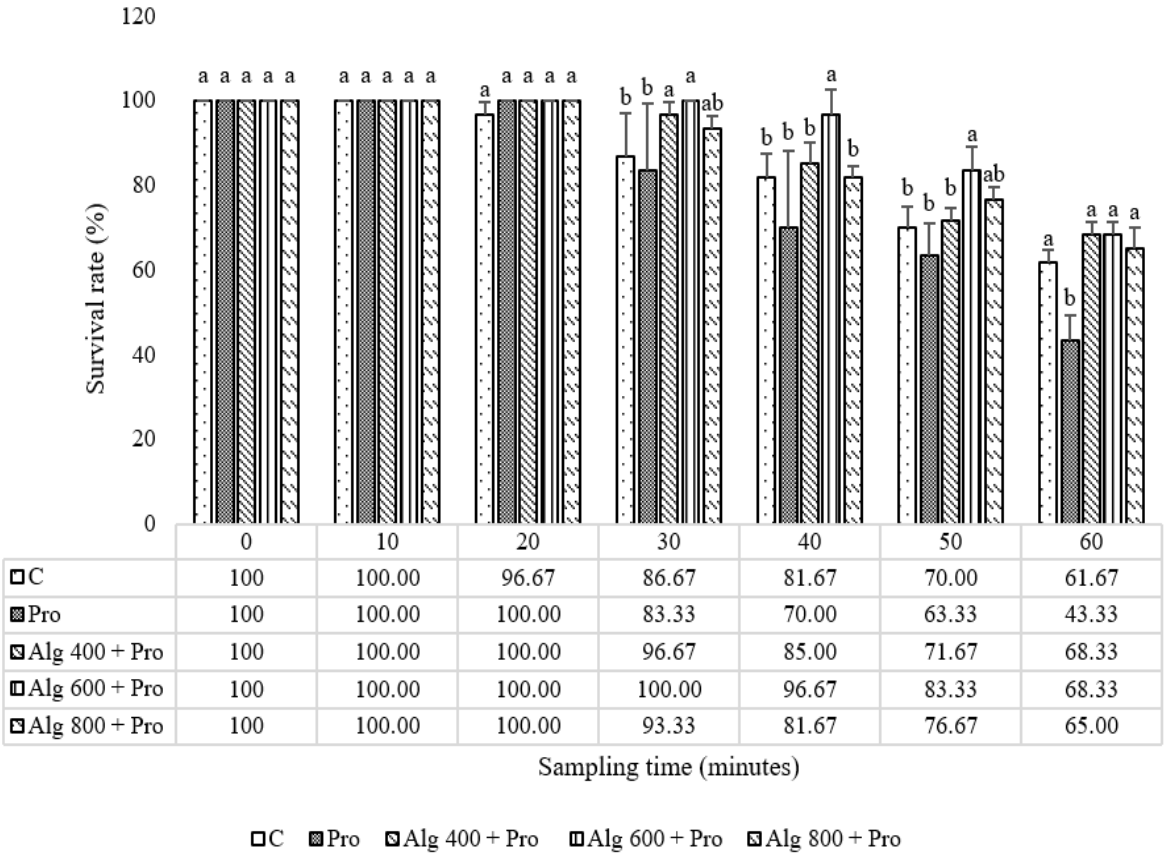
challenge, the PL fed on Artemia enriched probiotics treatments give the best results significantly.

V. parahaemolyticus was identified as the causative agent of acute hepatopancreatic necrosis which caused 100% mortality first 35 days of the post larva stage caused by severe hepatopancreas atrophy (Choi *et al*., 2017). The conventional techniques, such as antibiotics and disinfectants, have had limited success in preventing or curing

AHPND. Furthermore, their use has been linked to changes in the host gut microbiota and immunity, as well as the development of antibiotic resistance in bacterial pathogens. It was reported that *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* isolated from *L. vannamei* pond and *V. vulnificus* were resistance to some beta lactam (Ampicilin, Amoxicilin dan Co-Amoxiclav) antibiotics (Yano *et al*., 2011).

In this present research, the PL resistance to severe *VpAHPND* was reached from *L. vannamei* PL fed on Artemie and probiotics enrichment. Al-Nabulsi *et al*. (2022) noted that they discovered the bactericidal effect from novel exopolysaccharide from *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. Inspite of secreted exopolysaccharides, increase disease resistance and gut bacterial diversity, the presence of probiotics apparently improved midgut characteristics by enhancing microvilli and intestinal wall thickness and improved resistance to VpAHPND (Kewcharoen & Srisapoome, 2019).

Figure 4 depicted that *L. vannamei* PL fed on Artemia enriched with alginate 600 ppm and FNCC-004 probiotics treatment reached the highest survival rate at at 30; 40; and 50 minutes exposure (100; 96.67±5.77; 83.33±5.77%), respectively. The lowest survival rate is reached in PL fed on Artemia enriched with probiotics, solely at 40; 50; and 60 minutes exposure (70±18.03; 63±7.64; 43±5.77%) after stress salinity. In this present study, the difference of osmotic stress is extremely high, and the PL was administered the salinity shock from 25 ppt to 0 ppt. Based on Yudiati *et al*. (2020) and Sudaryono *et al*. (2018), the osmotic stress on marine cultivan is correlated to the immune system. In this present research, in relevance to the survival rate data (Figure 1), the increment of immune response occurs from *L vannamei* PL fed on Artemia-enriched with synbiotics of alginate and FNCC-004 probiotics. The certain dose of synbiotics resulted the best survival rate in acute osmotic stress due to salinity shock.



	0	10	20	30	40	50	60
C	100	100.00	96.67	86.67	81.67	70.00	61.67
Pro	100	100.00	100.00	83.33	70.00	63.33	43.33
Alg 400 + Pro	100	100.00	100.00	96.67	85.00	71.67	68.33
Alg 600 + Pro	100	100.00	100.00	100.00	96.67	83.33	68.33
Alg 800 + Pro	100	100.00	100.00	93.33	81.67	76.67	65.00

Figure 4. Survival rate of *L. vannamei* PL fed on different Artemia enrichment after 60 minutes stress salinity Different letter denotes significantly different at $p<0.05$

CONCLUSION

The synbiotics of alginate and *Lactobacillus bulgaricus* FNCC-004 and those sinergically mechanism resulted the best survival rate during 14 days of rearing, and best tolerance to osmotic stress. *Lactobacillus bulgaricus* FNCC-004 was able to give maximum protection against severe *Vibrio parahaemolyticus* AHPND and reached the highest resistance to severe *VpAHNPD*. The immune response is postulated to be interrelated to these results, even though, the weight gain is similar among treatments.

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